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Retinoic acid is required for the initiation of outgrowth in the chick limb bud

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Background: Retinoic acid (RA) is present in the chick limb bud, and excess RA induces limb duplications. Here, we have investigated the role of endogenous RA during chick limb development by preventing the synthesis of RA and testing the effect on various genes expressed during limb initiation and outgrowth.

Results: We demonstrate that the stage 20/21 limb bud synthesizes didehydroretinoic acid (ddRA), and that the posterior half of the limb bud synthesizes ddRA at a higher rate than the anterior half. Disulphiram inhibits this synthesis at micromolar concentrations. Administering disulphiram to embryos prior to limb bud outgrowth (stages 12–18) abolishes outgrowth, and no limb develops in the majority of cases. Disulphiram treatment also prevents the expression of *Sonic hedgehog* (*Shh*), but the expression of the fibroblast growth factor-8 gene (*Fgf*-8) appears as normal in the ectoderm over the prospective limb bud. The application of a bead soaked in RA can rescue *Shh* expression. Disulphiram treatment of later limb buds (stages 20–23) similarly down-regulates *Shh*, and also *Fgf*-4, expression, whereas the expression of *Fgf*-8, as at earlier stages, is initially unaffected. Again, RA can rescue the expression of *Shh* in these limb buds.

Conclusions: RA, in conjunction with *Fgf*-8, may be needed for the induction of the chick limb bud and the induction of *Shh* and *Fgf*-4 expression. The expression of *Shh* and *Fgf*-4 remains dependent upon the continued synthesis of RA within the limb bud. Didehydroretinoic acid is the major active retinoid in the stage 20 chick limb bud.

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Received: 19 April 1996
Revised: 13 June 1996
Accepted: 4 July 1996

Current Biology 1996, Vol 6 No 9:1124–1133

© Current Biology Ltd ISSN 0960-9822

Background

Retinoic acid (RA) plays an important morphogenetic role in several systems of the developing embryo. In the developing chick limb bud, for instance, the addition of excess RA has the remarkable ability to organize a new outgrowth from anterior tissue, and the resulting duplicated limb has a double posterior structure [1]. The subsequent finding that all-*trans*-RA is present endogenously in the chick limb bud at a higher concentration on the posterior side than the anterior side provided powerful support for the idea that RA is, in some way, responsible for organizing pattern in the anteroposterior axis of the limb bud [2].

In recent years, however, this simple concept has been complicated by three further advances in our understanding of limbs. Firstly, in the regenerating amphibian limb, RA can respecify all three limb axes (proximodistal, anteroposterior and dorsoventral) [3], rather than just one axis, as in the chick. Furthermore, complete extra limb fields can be induced from tail tissue in the frog tadpole [4,5] and from posterior tissue in the mouse embryo [6].

The second advance is that several other retinoic acids, in addition to all-*trans*-RA (tRA), have been shown to be morphogenetically active. These include 9-*cis*-RA, 4-*oxo*-RA and didehydroretinoic acid (ddRA) [7–9]. Although 9-*cis*-RA induces duplications of the chick limb bud with a greater potency than tRA [7], this isomer cannot be detected endogenously in either chick or mouse limb buds [10,11], nor in any other part of the mouse embryo (C.H. and M.M., unpublished data). The wound epidermis of the regenerating amphibian limb synthesizes 9-*cis*-RA [12], so this may reflect a species-specific difference, but this isomer is unlikely to play a significant part in chick limb bud development. 4-*oxo*-RA is present in the *Xenopus* embryo [8] but not in the zebrafish embryo [13], and we know nothing about its presence in higher vertebrate embryos. On the other hand, the chick limb bud contains 4–6-fold higher concentrations of ddRA compared with tRA [9,10], and ddRA is equipotent at inducing duplications in the chick limb bud [9]. Thus, this molecule complicates the picture as its endogenous concentration would swamp the endogenous gradient of tRA, and measurements of the concentration of ddRA between anterior and posterior parts of the chick limb bud indicate only a very small excess (1.15-fold more) in the posterior part [10]. Interestingly, despite the prevalence of ddRA in the chick limb bud, it cannot be detected in the mouse limb bud [10], again suggesting

species-specific differences in retinoid metabolism. Here, we ask which type of RA is synthesized by the chick limb bud, and whether the anterior and posterior halves of the limb bud differ in rates of synthesis.

The third advance is that recent molecular studies of the developing limb bud have identified an amazing array of genes involved in limb development — the fibroblast growth factors (*Fgf*s) [14–17], *Sonic hedgehog* (*Shh*) [18], the *Hoxd* complex [19], the *Hoxa* complex [20,21], *Hoxb*-8 [22], the bone morphogenetic proteins (BMPs) [23], the *Msx* genes [24], *Wnt*-7a [25] and *Lmx*-1 [26]. Analysis of the role of some of these genes — particularly *Fgf*-4, which is expressed in the apical ectodermal ridge (AER) and which can substitute for the functions of the ridge, *Shh*, which is expressed in the region known as the zone of polarizing activity (ZPA), and *Wnt*-7a, which is expressed in the dorsal ectoderm — has suggested that they have a complex reciprocal relationship [25,27–29]. RA is generally assumed to be involved in the induction of limb outgrowth and, by implication, in the induction of some of these genes, but not in their maintenance of expression during later development.

Rigorous testing of these ideas has not been possible, however, because of the difficulty in depleting embryos of RA (unlike the readily available 'knockout' technology for the creation of mutants which do not express a particular gene). Here, we test the role of endogenous RA both in limb induction and in subsequent outgrowth using disulphiram, a compound which inhibits the action of aldehyde dehydrogenases and, consequently, the synthesis of RA. Disulphiram prevents the initiation of the chick limb bud and the appearance of *Shh* in the posterior mesenchyme, but not the appearance of *Fgf*-8 in the AER. *Shh* expression can be rescued with RA, demonstrating a role for endogenous RA in the early events of limb initiation. We show that there is a difference in the rate of synthesis of RA between anterior and posterior halves of the limb bud, and that ddRA is the main form of RA synthesized. Furthermore, during later limb bud outgrowth, similar results are obtained: *Shh* expression and *Fgf*-4 expression in the AER both depend upon the synthesis of RA in the limb bud, whereas *Fgf*-8 does not become down-regulated immediately. These results demonstrate a continuing role for RA in limb development, in addition to its role in limb initiation.

Results

Synthesis of RA by chick limb buds

We first asked which type of retinoic acid was synthesized from [^{3}H]all-*trans*-retinol in the chick limb bud. A two-column high-pressure liquid chromatography (HPLC) procedure was used to identify which [^{3}H]RA isomers were generated after incubating stage 20/21 limb buds with [^{3}H]all-*trans*-retinol. (We have recently developed and used this method to demonstrate that the wound epidermis of the regenerating newt limb synthesizes 9-*cis*-RA [12].) After incubating 50–80 limb buds in 500 nM [^{3}H]retinol for 5 hours, extracts were fractionated on a normal-phase LiChrospher NH_2 column, and the acid metabolites collected (Fig. 1a) [30]. This column retains only the acids and discards excess unmetabolized [^{3}H]retinol, which obscures other retinoid peaks on a reverse-phase chromatograph. This acid peak was then re-run on a C_{18} reverse-phase column, which can separate different retinoic acids, revealing that the label was in a peak corresponding to ddRA (Fig. 1b). Thus, it seems that the chick limb bud metabolizes all-*trans*-retinol primarily to ddRA, and not to all-*trans*-RA (tRA) as might be expected. Other retinoic acids, including tRA, may be generated but, if so, they were at undetectable levels — any peak 5–10-fold lower than the ddRA peak would not have been detected.

These observations are consistent with previous data from the chick: the endogenous concentration of ddRA is 5–6-fold higher than that of tRA; chick tissue contains the enzymes required for the synthesis of ddRA from retinol; and there is no significant conversion of ddRA to tRA [9]. Furthermore, the results are not artefacts of the method as, using the same techniques, we have found that the newt wound epidermis generates 9-*cis*-RA [12] and mouse embryonic tissue generates all-*trans*-RA (M.M. and C.H., unpublished data). That different organisms generate different retinoic acids may have mechanistic significance for the differential activation of receptor pathways.

Differential synthesis of ddRA by anterior and posterior halves of the chick limb bud

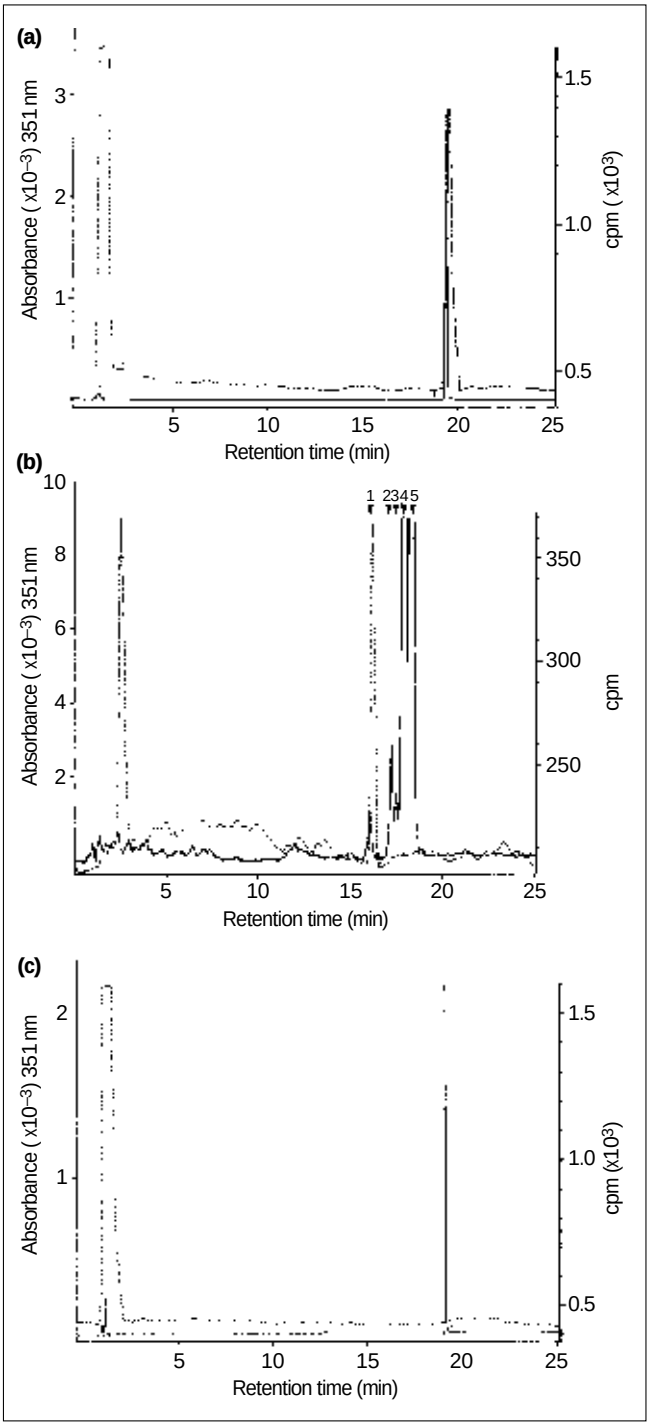
The observation of the synthesis of ddRA by the limb bud raises the question of the significance of the differential distribution of tRA between anterior and posterior portions of the limb bud [2], as this must now be relegated to being only a minor component of the retinoid profile of the limb bud. To examine whether differences in the synthesis of ddRA could be detected between anterior and posterior halves of limb bud, we cut 150–200 stage 20/21 buds into two halves and repeated the incubations with [^{3}H]retinol followed by chromatography.

The results of three repeats of this experiment showed that there was a small, but reproducibly higher rate of synthesis of ddRA by posterior halves compared with anterior halves of the limb bud (Table 1). These data are supported by the small difference in endogenous concentration of ddRA between anterior and posterior halves of the chick limb bud [10] and by similar measurements that have been made using the F9 reporter cell system (M.M., unpublished data).

Inhibition of synthesis by disulphiram

Disulphiram is a compound which inhibits the action of aldehyde dehydrogenase enzymes [31] and, consequently,

inhibits the synthesis of RA in embryonic systems [32,33]. When limb buds were incubated with [^{3}H]retinol, in the presence of 1–10 μM disulphiram, the synthesis of acid metabolites was completely inhibited (Fig. 1c). This was a dose-dependent phenomenon that showed a cut-off point between 10^{-5} M and 10^{-6} M. This concentration is the same as that at which disulphiram inhibits the synthesis of RA from retinaldehyde in the chick, mouse and rat retina (6–50 μM) [32,33].



The effect of disulphiram on early limb development

Disulphiram could therefore now be used to examine the effect of inhibiting RA synthesis on limb development. In the first series of experiments, disulphiram was incorporated into Silastic Medical Elastomer, a compound which can be used as a slow release depot [34]. A $250 \times 250 \times 500$ μm block of disulphiram-impregnated silastin (25–100 mg ml^{-1}) was then placed adjacent to the flank of pre-limb bud or early limb bud (stages 15–18) and the limbs observed at daily intervals.

This treatment resulted in three different outcomes. In the majority of cases (76 %, $n = 43$; Table 2, row 1), no limb developed on the treated side (Fig. 2a,d,e) or a small protrusion appeared on the flank in the region of the posterior part of the limb bud, presumably in an attempt to initiate development (Fig. 2c). Sections through these embryos confirmed the lack of any recognizable limb outgrowth (Fig. 2d). When these embryos were incubated until good cartilage differentiation had occurred in the contralateral limb, all that was present on the treated side was a scapula and sometimes part of a humerus as well (Fig. 2e). In such inhibited limbs, the shoulder girdle always formed.

The remaining 24 % of cases had normal limbs or displaced limbs. We attribute the small number of normal limbs to a failure of the experiment — the block of silastin had been displaced during incubation, and so these embryos never received any treatment. We attribute displaced limbs to either a recovery from the effect of disulphiram or a partial displacement of the silastin block such that the dose received was less than intended. These displaced limbs, as

Figure 1

HPLC chromatograms showing the synthesis of ddRA from retinol by chick limb buds and its inhibition by disulphiram. **(a)** NH_2 column chromatograph of limb-bud extracts incubated with [^{3}H]all-*trans*-retinol. This column retains acids, separating synthesized [^{3}H]RAs from the added retinol. The dotted line shows the radioactivity (scale on the right) with a large peak in the void volume (unmetabolized [^{3}H]retinol) and a peak at 19.5 min, which is the retained [^{3}H]RA. This has been generated from the added retinol, as control incubations without any tissue does not give a RA peak. The solid line is the UV absorbance of cold RA which was added to the extract, thereby confirming the identity of the radioactive peak. **(b)** When the fractions from the NH_2 column are collected and re-run on a C_{18} reverse phase column, the identity of the [^{3}H]RA isomer can be deduced. The solid line shows the UV absorbance of 5 cold standards which are run along with the radioactive sample: 1 = ddRA, 2 = 13-*cis*-RA, 3 = 11-*cis*-RA, 4 = 9-*cis*-RA, 5 = all-*trans*-RA. The dotted line is the radioactive fractions from the NH_2 column, which coelute with ddRA. The radioactive peak at 2.5 min is a highly polar RA metabolite which elutes in the void volume; it is not any known polar metabolite, such as 4-*oxo*-RA, which elutes at 7.5 min. **(c)** Limb buds incubated with 10^{-6} M disulphiram and the extract run on a NH_2 column. In this case, the synthesis of retinoic acid is completely inhibited and the only radioactive peak (dotted line) is the unmetabolized [^{3}H]retinol in the void volume. Solid line marks the UV absorbance of a cold RA standard.

Table 1

Rates of synthesis of ddRA (in cpm per mg tissue) of anterior and posterior halves of stage 20/21 chick limb buds.

	Experiment		
	1	2	3
Anterior	73	87	140
Posterior	101	116	173
A:P ratio	1:1.4	1:1.33	1:1.24

Table 2

Effects of disulphiram treatment on stage 15–18 chick embryos.

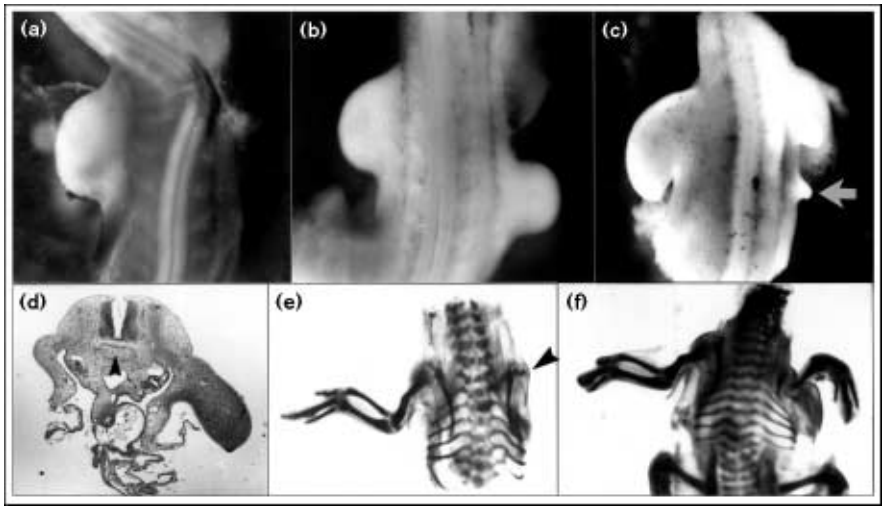
Dose of disulphiram (mg ml^{-1})	Number of cases	Inhibition of limb initiation/outgrowth	Absence of *Shh* expression		
			24 h	36 h	48 h
100	43	33 (76%)	–	–	–
25	5	3 (60%)	–	–	3
10	6	4 (67%)	–	4	–
10–25	12	9 (75%)	9	–	–

shown in Figure 2b, were always displaced posteriorly, never anteriorly, and by as much as a four-somite distance. When these limbs were incubated until good cartilage differentiation had occurred, the displacement became obvious and the limbs were always deficient in cartilage structure (Fig. 2f). However, these limbs all had the appropriate anteroposterior polarity, even though they were displaced posteriorly, unlike the situation where an extra limb is induced posteriorly to the normal one — in which case the anteroposterior polarity of the induced limb is reversed.

Two other methods used to administer disulphiram produced identical results to the disulphiram-impregnated silastin treatment. One method involved soaking a piece of newsprint in disulphiram solutions, and the other involved injecting the lipophilic dye DiI and disulphiram into the appropriate position in the flank of the embryo. Summing all the data with three different methods of disulphiram administration gives a total of 70 % inhibition of limb outgrowth ($n = 81$) at these early stages. Control treatments using silastin blocks, dimethylsulphoxide (DMSO)-soaked paper or injections of DMSO and DiI had no effect on limb development.

Although a complete dose-response experiment has not been performed, it was observed that far fewer limbs were affected by a dose of disulphiram below 10 mg ml^{-1}. The dose of disulphiram administered *in vivo* (100 mM) is considerably higher than that required to inhibit RA synthesis *in vitro* (10–100 μm). We attribute this to the fact that the embryos were treated with disulphiram externally, which would result in only a very small proportion of the dose being taken up into the tissues or, when injected, a very small area of the flank being treated. Three observations

Figure 2



The effect of disulphiram on chick limb development. **(a)** A block of silastin impregnated with disulphiram (100 mg ml^{-1}) was placed next to the right flank of a stage 15 embryo. After 48 h, the left limb bud has appeared normally, but the right limb bud is completely absent. **(b)** A similar treatment to (a) has, in this case, resulted in a smaller limb bud on the right side which has shifted posteriorly by about 4 somites. **(c)** A similar treatment to (a) has, in this case, resulted in only a pimple of tissue appearing in the posterior region of the location of the limb bud (arrow). **(d)** Transverse section through an embryo showing a normal limb bud on the right side of the section and the complete absence of a limb on the disulphiram-treated left side of the section. Also present in these sections is an abnormal notochord (arrowhead), which has become zig-zagged in the region of disulphiram treatment. This is the same phenomenon that is seen in zebrafish embryos [13]. **(e)** Victoria blue-stained 9 day embryo, which had been grown up from a group shown in (a). The control (left) limb has a normal cartilage pattern, whereas the disulphiram-treated (right) limb only has the shoulder girdle. The limb is completely missing on this side (arrowhead). **(f)** Victoria blue stained 9 day embryo which had been grown up from a group shown in (b). The control (left) limb has a normal cartilage pattern, whereas the disulphiram-treated (right) limb has been shifted posteriorly by a distance of about 3–4 vertebrae. The limb itself is very deficient, the humerus is stunted, the radius is missing and the ulna is stunted.

argue against a simple toxic effect of disulphiram despite the relatively high doses administered: the limbs could be rescued with RA (see below); *Fgf*-8 continued to be expressed in the AER (see below); and the effects on gene expression could be reproduced in culture at doses of 10–100 μM (data not shown).

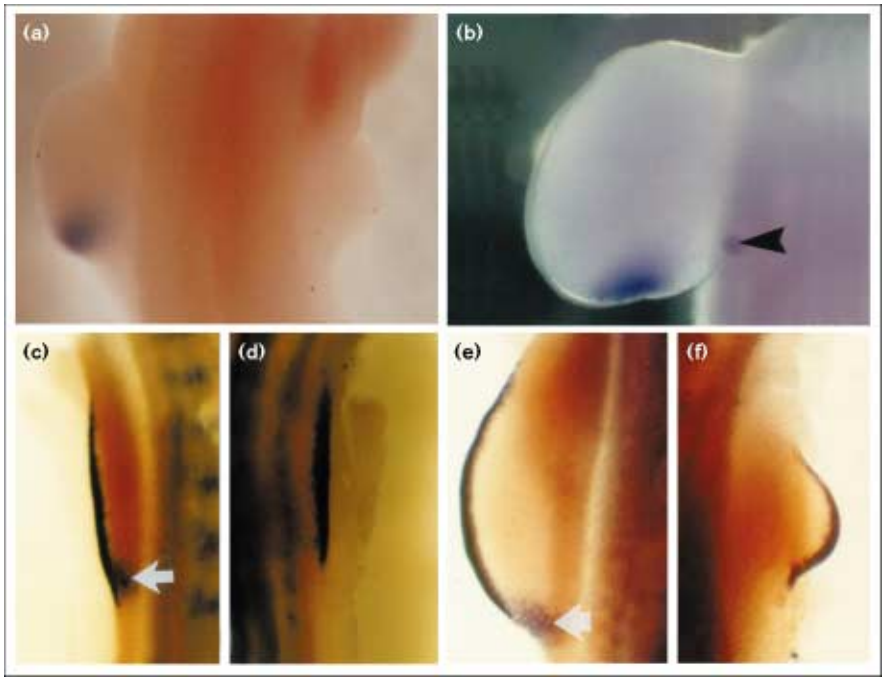
Effect of disulphiram on gene expression at early stages

We analyzed the effect of disulphiram on two genes expressed in the early limb bud, *Shh* and *Fgf*-8. *Shh* is expressed in the mesenchyme of the posterior margin of the limb from stage 17 [18]; by this stage, limb outgrowth is clearly visible. As ectopic *Shh* is inducible by RA [18], we wished to ask whether endogenous *Shh* is responsive to RA synthesis. *Fgf*-8 is expressed in the ectoderm over the prospective limb bud at stage 16, and is thought to play a role in the initiation of limb outgrowth [17]. The range of stages (15–18) at which disulphiram treatment was performed initially was the same as in the previous experiment, and encompassed the period over which *Shh* and *Fgf*-8 begin to be expressed in limb tissue.

Treatment with disulphiram prevented *Shh* expression in the area of the missing limb when examined 24 hours later (Fig. 3a; Table 2, row 4), 36 hours later (Table 2, row 3) or 48 hours later (Table 2, row 2). In the minority of cases which showed good limb outgrowth, *Shh* expression was normal. The frequency of loss of *Shh* expression in these experiments (69 %, $n = 23$) was the same as the frequency of loss of limbs (70 %, $n = 81$) in earlier experiments. The earliest time period at which an effect of disulphiram on expression of *Shh* was observed was 17 hours (data not shown), so *Shh* does not respond rapidly to the inhibition of RA synthesis. At the other extreme, as the frequency of loss of *Shh* expression at 48 hours was the same as at earlier time periods, we presume that limb buds cannot recover from an early loss of *Shh*, nor regenerate *Shh* expression.

In contrast, *Fgf*-8 expression appeared normal in the ectoderm over the initiating limb in the absence of RA synthesis and *Shh* expression, as shown by double *in situ* hybridization experiments (Fig. 3c,d). The absence of *Shh* expression in these *Fgf*-8-positive limb buds confirmed that the disulphiram treatment had been effective. In order to ensure that we had treated embryos considerably before the onset of *Fgf*-8 expression in the ectoderm of the limb field, we performed additional experiments treating as early as stage 12, and examined the embryos by double *in situ* hybridization 24 hours later. In this case, 8 out of 17 limbs (47 %) showed *Fgf*-8 expression in the absence of *Shh* expression. The remaining cases showed expression of both genes with good limb outgrowth, and were presumably failed experiments. At longer time periods after treatment (36 hours), we observed continued expression of

Figure 3



Shh and *Fgf*-8 expression in disulphiram-treated early limb buds. **(a)** *Shh* expression in an embryo which had been injected with disulphiram (50 mg ml^{-1}) 24 h earlier into the flank at stage 16 on the right side. On this side, the limb bud has not developed and there is no *Shh* expression, whereas the control (left) side shows typical *Shh* expression in the ZPA. **(b)** A disulphiram-treated early embryo (stages 15–18), which was rescued by implanting a RA-soaked bead opposite somite 20 (arrowhead). In contrast to disulphiram treatment, which prevents outgrowth of the limb and *Shh* expression, the addition of RA has allowed the limb to grow out and express *Shh.* **(c,d)** Double *in situ* hybridization with *Shh* and *Fgf*-8 in a stage 18 embryo. (c) The normal left limb shows *Shh* expression on the posterior margin (arrow) and *Fgf*-8 in the AER. (d) The right limb had been treated 24 h earlier (at stage 15) with disulphiram and, despite showing no *Shh* expression, still shows *Fgf*-8 expression even though no limb outgrowth is visible. **(e,f)** Expression of *Fgf*-8 in a stage 22 embryo. (e) The normal left limb shows *Fgf*-8 expression in the AER and *Shh* expression in the ZPA (arrow). (f) The right limb had been treated 36 h earlier (at stage 15) with disulphiram and shows only a very small amount of outgrowth despite the continued expression of *Fgf*-8.

Fgf-8 despite the severe inhibition of limb outgrowth (Fig. 3e,f). After 36 hours, *Fgf*-8 expression began to decline.

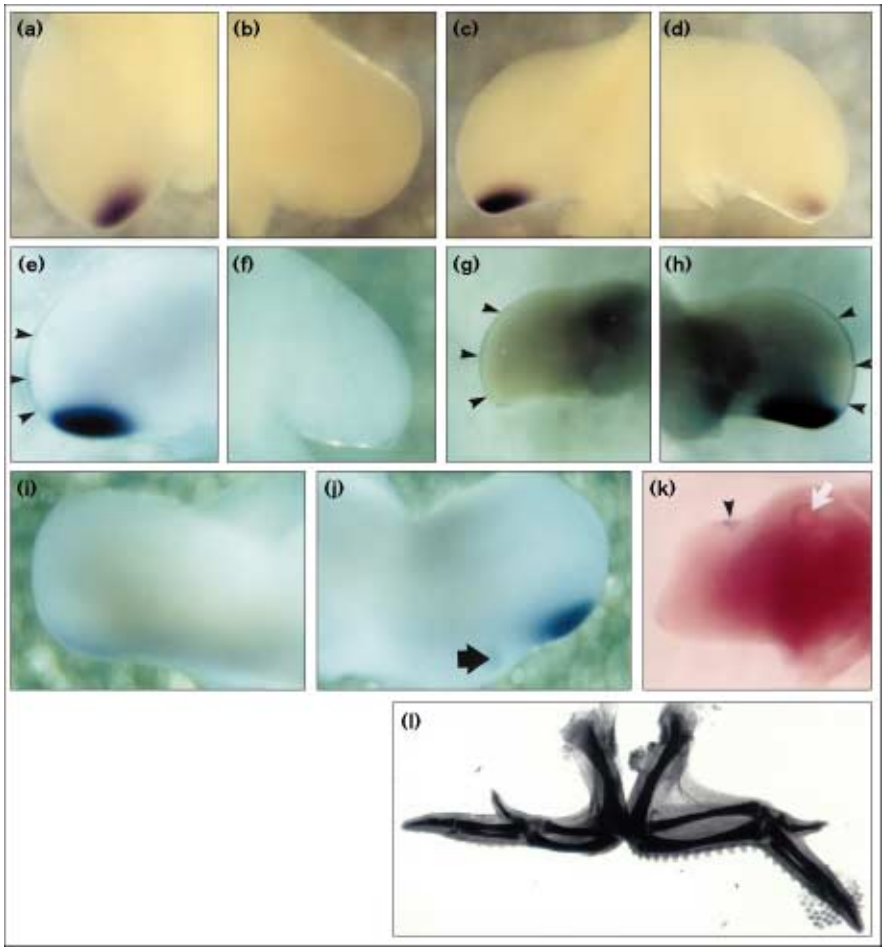
Rescue of limb outgrowth by RA

If the effect of disulphiram *in vivo* is to inhibit RA synthesis (along with other aldehyde dehydrogenases), resulting in the failure to induce limb outgrowth, then the provision of additional RA should restore limb outgrowth. If, however, its effect is toxic, killing cells, then no such rescue would be expected (although the initial lack of effect on *Fgf*-8 expression already argues against this contention). To distinguish between these two alternatives, we treated embryos at the same stages (stages 15–18) with disulphiram and RA simultaneously. Beads soaked in RA (at concentrations of 100 $\mu g\,ml^{-1}$ to 1 $mg\,ml^{-1}$) were placed opposite somite 20, and the flank of the same side of the embryo was treated with disulphiram. Rescue was assayed by the presence of a high level of expression of *Shh* and by obvious outgrowth. After 24 hours, 75 % of embryos showed a high level of *Shh* expression (n = 8; Fig. 3b), and after 36 hours, 60 % showed a high level of *Shh* expression (n = 5). Thus, 64 % of disulphiram-treated limb buds expressed *Shh* at high levels after rescue by RA. Furthermore, most of the remaining limbs showed some rescue of outgrowth and low *Shh* expression. In the initial experiments, disulphiram alone abolished *Shh* expression in 69 % of embryos (Table 2; thus 31 % showed some *Shh* expression); in these rescue experiments, 64 % of limbs expressed high levels of *Shh*. Hence, the addition of RA more than doubled the number of disulphiram-treated limb buds in which *Shh* was expressed.

Effect of disulphiram on gene expression at later stages

To determine whether inhibition of RA synthesis has any effect on gene expression in limb buds that have become established, limb buds at stages 20–23 were treated with

Figure 4



Shh, *Fgf*-4 and *Fgf*-8 expression in disulphiram-treated late limb buds. (a,b) *Shh* expression in an embryo whose right limb bud (b) had been injected with disulphiram (50 $mg\,ml^{-1}$) 24 h earlier at stage 20. There is no *Shh* expression in this limb. The left control limb, shown in (a), has normal *Shh* expression. (c,d) A similar embryo to that shown in (a,b), except that, in this case, a small and weakly expressing region of *Shh* has remained in the disulphiram-injected right limb (d). Normal expression is present in the control left limb (c). (e,f) Double *in situ* hybridization experiment with *Fgf*-4 (in the AER; arrowheads) and *Shh* in a control limb (e) and a disulphiram-treated limb (f). In the disulphiram-treated limb, neither gene is expressed, in contrast to the control. (g,h) Double *in situ* hybridizations with *Fgf*-8 (in the AER; arrowheads) and *Shh* in a disulphiram-treated limb (g) and a control limb (h). In the disulphiram-treated limb, *Shh* is absent but *Fgf*-8, although weaker, has continued to be expressed in the AER. (i,j) Late rescued limbs. (i) A control limb, 48 h after stage 20–22, has reached stage 28 and *Shh* expression is much reduced on the posterior margin. (j) In contrast, a disulphiram-treated limb with an RA-soaked bead placed posteriorly (arrow) has resulted in the rescue and continued strong expression of *Shh*. (k) A stage 20 disulphiram-treated limb 30 h after an RA bead (white arrow) was placed anteriorly. The normal expression of *Shh* on the posterior margin has disappeared, and a new domain of *Shh* expression is beginning to appear on the anterior side (arrowhead). (l) The cartilage pattern in a limb which had been treated with disulphiram at stage 20–22 and incubated for 7 days (on the left) compared with its normal counterpart (on the right). Despite the loss of *Shh* and *Fgf*-4 expression following administration of disulphiram, as shown in panels (a–h), the anteroposterior patterning of the limb on the left is perfectly normal although the proximodistal length of the elements is reduced (see text for details).

disulphiram. The expression of *Shh* and *Fgf*-8 was examined as in the previous section, together with *Fgf*-4, which is thought to be involved in a feedback loop with *Shh* and *Wnt*-7a [25,27–29]. 24 hours after treatment, the expression of *Shh* was either switched off (Fig. 4a,b) or severely down-regulated (Fig. 4c,d) in 48 % of limbs (n = 60; Table 3). Similar results were also seen 30 hours (38 % inhibition, n = 13) and 36 hours (38 % inhibition, n = 8) after treatment. Treated limb buds were then examined for the expression of both *Shh* and *Fgf*-4, or *Shh* and *Fgf*-8, by double *in situ* hybridization. The results revealed that, in those limb buds where *Shh* was down-regulated, so also was *Fgf*-4 expression in the AER (Fig. 4e,f). We interpret these results as confirmation of the feedback loop which is proposed to operate between these two genes [27,28]. In contrast, *Fgf*-8 expression was maintained in the AER, although at somewhat reduced levels (Fig. 4g,h). This demonstrates that, as at earlier stages, *Fgf*-8 expression is not immediately responsive to the absence of RA synthesis.

The majority of limb buds treated with disulphiram at these later stages, when observed externally, seemed relatively normal. However, 47 % (n = 64; Table 3, row 2) were observed to show either a slight distal thinning of the limbs or a reduction in the length of the proximodistal axis when compared with the contralateral control limb bud. A group of these limbs (n = 29) were incubated for a total of 10 days to examine the size of the cartilage elements (Table 3, row 3). All these limbs had normal anteroposterior polarity, although 30 % showed slight reductions in the length of both the humerus (range 0–17 %) and the radius (range 5–25 %) (Fig. 4l). Control treated limbs showed no reduction in *Shh* expression, no change in external shape and normal cartilage patterning at 7–10 days of incubation.

Rescue of later limb buds by RA

In order to demonstrate that the action of disulphiram on gene expression at later stages was not due to toxicity, we performed rescue experiments of disulphiram-treated limbs (stage 20–22), using RA beads (soaked in 100 μg ml^{-1} to 1 mg ml^{-1} disulphiram) placed under the AER at the posterior margin. This treatment resulted in high levels of expression of *Shh* 24 hours (78 %, n = 14), 32 hours (100 %, n = 2) and 36 hours (80 %, n = 5; Fig. 4i,j) after treatment. Without rescue, 52 % of limbs showed strong *Shh* expression whereas, with added RA, this frequency increased to 81 %. Control beads soaked in DMSO and placed on the posterior margin of disulphiram treated limb buds did not rescue *Shh* expression.

Finally, we performed induction experiments by implanting a RA bead on the anterior margin of a disulphiram-treated limb rather than the posterior location as above. In 3 out of 12 cases, this treatment resulted in the disappearance of endogenous *Shh* on the posterior side (caused by disulphiram) and the appearance of *Shh* on the anterior side (caused by RA) (Fig. 4k).

Table 3

Effects of disulphiram treatment on stage 20–23 chick limb buds.

Dose of disulphiram (mg ml^{-1})	Number of cases	Reduction in *Shh* expression at 24 h	Change in shape of limb bud (24–48 h)	Normal cartilage pattern (7–10 days)
10–100	60	29 (48%)	–	–
10–100	64	–	30 (47%)	–
10–100	29	–	–	29 (100 %)

Discussion

RA synthesis in the chick limb bud

We have shown that the stage 20/21 chick limb bud synthesizes primarily didehydroretinoic acid from [^{3}H]all-*trans*-retinol in our assay system. In other experiments using the same procedures, we have found that the newt wound epithelium generates 9-*cis*-RA [12] and that the mouse embryo generates all-*trans*-RA. This suggests that the form of RA generated in embryos is species-specific, which may have consequences for the differential activation of specific receptor pathways. The retinoic-acid receptors (RARs) are activated by ddRA and tRA, but the other class of retinoic-acid receptors, the RXRs, are activated by 9-*cis*-RA [35]. Thus, in the absence of any detectable 9-*cis*-RA in the chick and mouse embryo, it is possible that RXRs are not activated by ligand and behave as silent partners. In the amphibian limb, however, where 9-*cis*-RA is the predominant retinoid synthesized, RXRs would be readily activated.

Although the endogenous concentration of ddRA in the chick limb bud is 4–6-fold higher than that of tRA [9,10] and we found ddRA to be the major metabolite, these two retinoic acids have identical abilities in activating the RARs [35]. Thus, these findings do not have any significance for receptor studies in the chick limb bud. We also demonstrated that the rate of synthesis of ddRA varied between the anterior and posterior half of the limb bud, with the posterior half showing a consistently higher rate than the anterior half. These data support the demonstration of a higher endogenous concentration of ddRA in the posterior half of the chick limb bud [10].

We showed here that disulphiram, an inhibitor of aldehyde dehydrogenases [31], inhibits the synthesis of retinoic acid and that this compound can be used (and has been used [32,33]) to ask questions about the role of endogenous RA in various embryonic systems. Of course,

other aldehyde dehydrogenases will be inhibited at the same time as there is no suggestion that disulphiram is specific for retinaldehyde dehydrogenase, but by performing rescue experiments we were able to demonstrate that it is the loss of RA itself which results in the failure of limb outgrowth and the down-regulation of certain genes.

RA and limb induction

The treatment of embryos with disulphiram before the limb bud appears prevents limb development in the majority of cases (70 %, $n = 81$). After 24 hours, a small pimple of tissue can be detected which does not express the gene *Shh*. By contrast, the expression of *Fgf*-8, which is present in the ectoderm over the prospective limb area, is normal in treated embryos, and continues to be expressed in inhibited limbs which fail to grow out. Eventually, *Fgf*-8 seems to disappear. That this effect of disulphiram is related to RA and not to a toxic effect on cells was demonstrated by the ability of added RA to rescue *Shh* expression in disulphiram-treated embryos. It is likely, therefore, that *Fgf*-8 and RA are both required for chick limb bud initiation, and that *Shh* expression is induced either by RA alone or by RA and *Fgf*-8. Thus, the induction of limb outgrowth by various FGFs [17,36] should only occur in regions of the embryo which can also synthesize RA.

Interestingly, some of these disulphiram-treated embryos did produce limbs, but these limbs were often displaced posteriorly, perhaps in an attempt to 'regenerate'. As the limb field encompasses a larger area that the tissue from which the normal limb actually arises, and limb induction can occur posterior to the normal limb [17,36], this posterior displacement may not be surprising. However, in contrast to the induced extra limbs, these displaced limbs had a normal anteroposterior polarity with respect to the body axis.

RA and later limb outgrowth

After the limb bud has begun development, disulphiram had no dramatic effect on final limb morphology. This striking difference in result between treating at stages prior to limb bud outgrowth compared with treating at stages after limb bud outgrowth is precisely the same as the effects of retinoid receptor antagonists on limb development [37]. Only minor abnormalities in limb structure were observed, such as proximodistally shorter elements, and the limbs were normal in the anteroposterior axis. Gene expression was affected in the same way as in the study on earlier stages, although in a smaller number of cases, and we also examined the effect on *Fgf*-4. *Shh* and *Fgf*-4 were both completely down-regulated by 24 hours, but *Fgf*-8 only showed a slight decrease in expression level. Again, the fact that the effect of disulphiram was not due to toxicity was demonstrated by adding back RA and reinitiating the expression of *Shh*. We could also reinitiate *Shh* expression on the anterior side of the limb bud having eliminated it on the posterior side (Fig. 4k).

We have not established whether both *Shh* and *Fgf*-4, or just one of these genes, are directly affected by levels of RA. Removal of the AER down-regulates *Shh* [29], so the down-regulation of *Fgf*-4 by lack of RA synthesis would also result in the absence of *Shh* expression. Similarly, ectopic *Shh* expression induces an expanded *Fgf*-4 domain [28], so if RA levels primarily control *Shh* expression, down-regulation of *Shh* will affect *Fgf*-4 as well. As RA and *Fgf*-4 act together to induce *Shh* [29], and neither will suffice alone, it is most likely that the lack of RA has its effect by interacting with both gene pathways. In reality, it is far too soon to speculate on the pathway(s) of RA action, as the other genes known to be involved in limb development (such as *Wnt*-7a, *Lmx*-1, the BMPs and the various *Hox* genes) need to be examined for the effect of disulphiram on their expression patterns.

In these experiments on later stage limb buds, the lack of major morphological effect, which has also been seen when treating limb buds with retinoid receptor antagonists [37], is in contrast to the effects on earlier stages. This may be due to one of several factors: the delay in the drug taking effect; the retention of some *Shh*-expressing cells (less than 100 ZPA cells are required for *Shh* to exert its function [38]); or the regeneration of *Shh*-expressing cells. However, we note that experiments involving the physical removal of the ZPA (*Shh*-expressing cells) after stage 20 revealed a similar lack of effect on the proximodistal or anteroposterior axes [39]. Also, the limb buds of the *limb deformity* (*ld*) mouse mutant fail to express *Fgf*-4, and the expression of *Shh* is considerably reduced, even though the limb abnormality is relatively minor — syndactyly and oligodactyly [40].

Developing chick limbs and regenerating amphibian limbs

These results bring studies on the role of RA in the chick limb bud and the regenerating amphibian limb much closer together. Emphasis in the chick experiments has always been on the role of RA in patterning across one axis, the anteroposterior axis. In the regenerating amphibian limb, where all three limb axes are affected [3], and in the regenerating amphibian tail, where RA transforms a tail blastema into legs [4,5], emphasis has been placed on the role of RA in the initiation of limb outgrowth — the establishment of the limb field [41]. The experiments described above suggest that, in the chick limb bud, RA is involved in limb induction, together with the FGFs. Clearly, it is now of interest to examine the effect of inhibiting RA synthesis with disulphiram on the expression of the multitude of other genes which are expressed in the developing limb bud. This compound provides us with a valuable tool to examine the role of RA, not only in the limb bud, but also in other embryonic systems where RA is thought to play a part.

Materials and methods

Chick embryos

Fertile eggs were obtained from Needle Farms, Enfield and incubated and staged according to Hamburger and Hamilton [42]. Eggs were windowed and treated with disulphiram (Sigma) by several methods. In one method, disulphiram powder was mixed with Silastic Medical Elastomer (Dow Corning) and the solidified mixture was cut up into small pieces (250 μm cubes). The cubes were placed adjacent to the flank on one side of the embryo at early stages (12–18) or underneath the limb bud at later stages (20–23). In another method, disulphiram was dissolved in DMSO and pieces of blotting paper were immersed in the solution. Fragments of these pieces were then pinned to the flank of the embryo above the limb bud or limb forming region. In another method, disulphiram was dissolved in dimethylformamide into which DiI had been dissolved and the mixture then injected into the posterior region of the limb bud. After varying time periods following treatment, embryos were fixed either for *in situ* hybridization or for cartilage staining with Victoria blue.

RA synthesis

50–80 stage 20/21 limb buds were incubated for 5 h in 1 ml of DMEM (Gibco) plus 500 nM [^{3}H]all-*trans*-retinol with various additives ($NaHCO_3$, penicillin/streptomycin, glutamine, BSA, transferrin, putrescine, sodium selenate, T3, insulin, progesterone, corticosterone) at 37 °C, 5 % CO_2. The same culture conditions were used for anterior *versus* posterior half limb buds, where 150–200 limb buds were cut into two halves.

HPLC

Retinoids were extracted from the limb buds (the supernatant was discarded) as described previously [2] with minor modifications. After sonication in 0.5 ml stabilizing buffer, 20 μl aliquots were removed for protein analysis and 200 ng each of the appropriate synthetic standards were added prior to extraction with two volumes of extraction solvent. The solvent phases were pooled after separation by low-speed microcentrifugation, dried under nitrogen, resuspended in 100 ml methanol, centrifuged at high speed and transferred to HPLC autosampler vials. HPLC was performed using a Beckman System Gold Hardware with a UV detector (351 nm) in series with a solid scintillant radioisotope detector. For normal phase chromatography, the method was according to [30], with modifications as follows: 50 μl of extract was autoinjected onto a 5 μm LiChrospher 100 NH_2 column (Merck) with an equivalent pre-column and eluted at 1ml min^{-1}, for 5 min initially, with 100 % mobile phase C (chloroform: methanol, 9:1) changing over 1 min to 100 % mobile phase D (chloroform: methanol: acetic acid, 9.0:0.9:0.1) for a further 20 min. The eluant was monitored both with a UV detector at 351nm and for radioactivity. The fractions corresponding to the RA peak were pooled, dried down resuspended in 100μl methanol and re-analyzed by reverse phase chromatography. For reverse-phase chromatography, the pooled peak fractions were autoinjected onto a 5 μm encapped C_{18} LiChrospher 100 column (Merck) with an equivalent pre-column and eluted at 1 ml min^{-1} under gradient conditions as follows: 40 % mobile phase A (1 % acetic acid), 60 % mobile phase B (acetonitrile: methanol, 3:1) rising linearly to 100 % mobile phase B over 25 min. The eluant was monitored as above.

In situ *hybridization*

These experiments were performed with *Shh*, *Fgf*-4 and *Fgf*-8 probes according to standard protocols.

Acknowledgements

We thank L. Niswander, C. Tickle, J. Dodd, M. Placzek and I. Mason for generous gifts of probes. T.S. is supported by a BBSRC studentship. This research was supported by Action Research (C.H.) and the Wellcome Trust to whom we are grateful for funding.

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